Genetic Ablation of Smoothened in Tumor-Associated Fibroblasts Promotes Pancreatic Tumorigenesis

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Abstract

Pancreatic cancer remains an overwhelmingly fatal disease with less than 5% of patients surviving beyond 5 years, largely due to our lack of understanding of the complexity of the disease. Many recent reports have begun to highlight the potential role that stromal cells—fibroblasts in particular—may have on pancreatic tumor cell biology and this report provides data that supports the theory of tumor-stroma co-evolution in pancreatic cancer. Here we use a novel mouse model to show that Smoothened (*Smo*) in the tumor-associated stroma suppresses pancreatic tumor initiation and development. We observed an increase in tumorigenesis events such as acinar-to-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN) lesions when *Smo* is conditionally deleted in pancreatic cancer associated fibroblasts (CAFs). To determine how *Smo* in fibroblasts is able to effect tumor progression, we harvested pancreatic CAFs from *Smo*-deleted and *Smo*-intact tumor bearing mice and performed microarray gene expression analysis. We found that *Smo* deleted CAFs had significantly altered the tumor suppressor PTEN, resulting in misregulated Phosphoinositide 3-kinase (PI3K) oncogenic pathway signaling. Thus, *Smo* ablation in pancreatic fibroblasts enhanced pancreatic tumor initiation primarily through loss of PTEN in fibroblasts. This report shows that deleting a gene in pancreatic fibroblasts causes a change in tumor-stroma co-evolution and that *Smo* is able to act

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*The research summarized herein has been conducted collaboratively. The primary author would like to graciously acknowledge and thank Xin Liu, Maria C. Cuitiño, Sarah Woelke, Stefan Trela, Raleigh Kladney, Lianbo Yu, Jinghai Wu, and Michael C. Ostrowski for their scientific contributions.

as a novel tumor suppressor in cancer associated fibroblasts to promote pancreatic carcinogenesis.

Introduction

Pancreatic cancer is an overwhelmingly fatal disease with approximately 95% of patients dying within 5 years of diagnosis [Siegel et al., 2013]. Moreover, our lack of knowledge in pancreatic cancer is evident in the fact that it has the least significant improvement in overall survival rate amongst all cancers over the last 35 years [Siegel et al., 2013]. Early studies on this disease focused on uncovering the genetics behind tumor formation, which led to the discovery of KRAS, CDKN4A, TP53, and SMAD4 mutations within tumor cells [Almoguera et al., 1998, Caldas et al., 1994, Redston et al., 1994, Hahn et al., 1996]. These studies were all performed in order to better understand the genetics behind Pancreatic Ductal Adenocarcinoma (PDAC), the most clinically prevalent form of pancreatic cancer. Recently, researchers have begun to study earlier events in pancreatic tumorigenesis and it has become apparent that PDAC originates from premalignant lesions termed Pancreatic Intraepithelial Neoplasms (PanIN) that arise from activating KRAS mutations in the epithelium [Hezel et al., 2006]. These efforts have also uncovered potential tumor initiating transformation events where pancreatic acinar epithelial cells trans-differentiate into ductal cells (termed acinar-to-ductal metaplasia, ADM) and then progress to PanIN [De La O et al., 2008]. Importantly, this transition from ADM to PanIN to PDAC is associated with a dramatic expansion of the pancreatic stroma, particularly in the fibroblast compartment. Thus far, the genetics of the tumor microenvironment during pancreatic tumor formation have been largely ignored. This report looks to further the understanding of the

role that the pancreatic stroma plays in epithelial transformation using genetically engineered mouse models.

The Hedgehog (HH) pathway is indispensable for normal development and often is inappropriately re-activated in many human cancers [Yauch et al., 2008 and Jiang et al. 2008]. HH ligands bind to the receptor Patched1 (PTCH1), releasing its' repression of Smoothened (SMO), the key component of the signaling cascade. Molecular and genomic analysis of human pancreatic tumors indicate that Hedgehog signaling is one of the core signaling pathways contributing to tumor malignancy [Tian et al., 2009 and Jones et al., 2008]. A prevailing view is that HH signaling in pancreatic cancer occurs in a paracrine manner leading primarily to activation of the pathway in stromal fibroblasts [Tian et al., 2009 and Bailey et al., 2009]. Consistent with this view, the reduction in tumor load upon SMO inhibition obtained in a GEM PDAC model was interpreted to indicate that stromal SMO inhibition could be a potential anti-tumor therapeutic [Oliver et al., 2009]. However, subsequent clinical trials based on these observations have failed in pancreatic cancer patients [Ruch et al., 2013]. More recently, ablation of Sonic Hedgehog (SHH) ligand in tumor cells or SHH co-receptors in fibroblasts was shown to decrease stromal activation and increase tumor cell growth [Rhim et al., 2014; Lee et al., 2014; Mathew et al., 2014]. These results bring into question the function of hedgehog signaling in the tumor stroma, and in particular in tumor fibroblasts, during pancreatic cancer progression.

Materials and Methods

Mice

The *Mist1-Kras*^{G12D}, *Smo*^{LoxP} (herein referred to as *Smo*^{fl}), and *Fsp-Cre* alleles have all been previously described [Tuveson et al., 2006, Long et al., 2001, and Trimboli et al., 2008]. The use of animals was in compliance with federal and University Laboratory Animal Resources (ULAR) at The Ohio State University regulations and was conducted under the protocol (2007A0120) which was reviewed and approved by the Ohio State University Institutional Animal Care and Use Committee (IACUC).

Tumor Studies

Mist1-Kras^{G12D} mice were sacrificed from 15-30 weeks after birth as indicated. At the time of euthanasia, total body weight and pancreas/tumor weight were documented. Pancreata, liver, and lung were removed and either frozen in OCT or fixed in formalin for 48-72 hours for subsequent histological processing by the Soild Tumor Pathology Core at OSU.

Pancreatic Cancer Associated Fibroblast Isolation

Mist1-Kras^{G12D} mice were euthanized and individual pancreata were removed and minced in 5mg/ml Type II collagenase in PBS. After mincing, pancreata were dissociated by shaking at 225 RPM at 37°C for 60 minutes. Cells were washed with complete Dulbecco's Modified Eagle Medium (DMEM, containing 10% FBS) and then pelleted by centrifugation at 200xg for 5 minutes at 4°C. Cell pellets were resuspended in 5ml complete DMEM and allowed to gravity precipitate at room temperature for 10 minutes. After precipitation, top 3ml of solution was discarded and cellular pellet was resuspended in up to 5ml of complete DMEM, thus constituting one round of gravity precipitation. An additional round of gravity precipitation was conducted and the final cellular pellet was seeded. 24-hours post seeding media was replaced with complete

DMEM. To further purify fibroblasts cultures, selective trypsonization was utilized where 0.25% trypsin was added to PBS-washed plated for 90 seconds and then fibroblasts were collected and re-seeded on a new plate. Fibroblast purity was confirmed by immunofluorescence as noted.

Immunofluorescence and Histological Staining of Tissue Sections

After removal from the body, tissue was immediately frozen in OCT or fixed in formalin for 48 hours, transferred to 70% ethanol and then paraffin embedded. Frozen or embedded tissue was subsequently sectioned and mounted on glass slides for further analysis. Sections were processed with xylenes and ethanol prior to DAKO antigen retrieval in a streamer for 30 minutes. Sections were blocked with DAKO protein block and tissues were stained with anti- F4/80 (Invitrogen), -vimentin (Cell Signaling), beta amylase (Cell Signaling), -cytokeratin 19 (Iowa Developmental Studies Hybridoma Bank), and -Ki67 (Abcam) as noted. Hematoxylin and eosin (H&E) and Masson's Trichrome staining was performed by the Solid Tumor Pathology Core at OSU.

Immunofluorescence Staining of Fibroblast Cultures

Cells were grown on glass cover slips to 90% confluence and fixed using 4% PFA. Fixed cells were blocked using DAKO protein block and stained with anti-α smooth muscle actin (Abcam) and –cytokeratin 19 (Iowa Developmental Studies Hybridoma Bank) as noted.

Western blotting

Primary pancreatic fibroblasts and were harvested by trypsinization and lysed in ice-cold RIPA lysis buffer, supplemented with 1mM PMSF, protease inhibitors (Amersham), and phosphatase inhibitor cocktails 1 and 2 (Sigma). Protein concentration was quantified using Bradford assay

(Sigma). Samples were fractionated by SDS–PAGE and transferred onto nitrocellulose membranes (Millipore), using the BioRad electrophoresis system (BioRad). Membranes were blocked in 5% nonfat dry milk in TBS-Tween (150 mM NaCl, 5 mM Tris-HCL ,pH 7.4), and blotted with primary antibodies: rabbit polyclonal antibody against SMO (1:500), Pten (1:1000), p-Akt (1:1000), total Akt (1:1000) from Cell Signaling Technology. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies, both used at 1:10000 dilution. Signal was developed with the ECL detection reagent (Amersham).

RNA Isolation and Quantitative Real Time PCR

RNA was isolated using Trizol reagent (Invitrogen), treated with DNase I (Ambion), and cDNA was generated using Superscript III Reverse Transcription (Invitrogen) as per the manufacturer's specifications. Quantitative real time PCR was performed using primer specific Roche Universal Probe Library system on Applied Biosystems Step One Plus real time PCR systems.

Gene Set Enrichment Analysis (GSEA)

GSEA v2.0 was downloaded from the BROAD institute (http://www.broadinstitute.org/gsea) and used to determine molecular pathways that were misregulated upon deletion of *Smo*. Gene sets were obtained from Broad institute GSEA and ToppGene Suite databases. Statistical analysis was determined using 1,000 random permutations of each gene set to obtain a nominal P value and normalized enrichment score (NES).

Results

Mist1-Kras^{G12D} Mice Developed Dramatic Stromal Reaction

Mist-Kras^{G12D} mice have been previously shown to develop mixed differentiated pancreatic carcinoma with the accompaniment of a rich stromal component, similar to human tumors [Tuveson et al., 2006]. We further characterized the *Mist1-Kras*^{G12D} mouse tumor model by performing a time-course analysis of the stromal reaction as the disease progresses. We monitored tumor histology on a bi-weekly basis and determined that 23 weeks of age consistently lead to increased collagen deposition in *Mist1-Kras*^{G12D} mice pancreata relative to wild type mice, as measured by Masson's Trichrome staining (Figure 1A-B). Histologically, 23-week old *Mist1-Kras*^{G12D} mice have developed PanIN and ADM lesions, but are still premalignant. Furthermore, we showed a dramatic increase in SMA+ fibroblasts and F4/80+ macrophages in 23-week old *Mist1-Kras*^{G12D} pancreata (Figure 1C-D). These data indicate that the *Mist1-Kras*^{G12D} mouse pancreatic tumor model is a valid system for us to study the role of pancreatic CAFs in tumor initiation and development.

Smo is Efficiently Deleted in Pancreatic Cancer Associated Fibroblasts

In order to conditionally delete *Smo* in pancreatic fibroblasts in the *Mist-Kras*^{G12D} pancreatic tumor model, we generated *Mist1-Kras*^{G12D}*; Fsp-Cre; Smo*^{fl} experimental mice and *Mist1-Kras*^{G12D}*; Smo*^{fl} control mice. Using a novel procedure for harvesting pancreatic fibroblasts (Figure 2A), we were able to isolate over 99% pure populations of fibroblasts as measured by the expression of fibroblast marker SMA and lack of expression of epithelial marker cytokeratin 19 (Figure 2B). Furthermore, we showed that these cells also expressed alternative fibroblast marker vimentin and lacked expression of cytokeratin 8 (data not shown). After verifying that novel method of purifying fibroblasts was effective, we showed that *Smo* was deleted exclusively in pancreatic fibroblasts from *Mist1-Kras*^{G12D}*; Fsp-Cre; Smo*^{fl} mice by western blot analysis

(Figure 2C). In order to show that the *Smo* was also excised *in vivo*, we stained formalin fixed paraffin embedded (FFPE) pancreatic tissue sections from *Mist1-Kras^{G12D}; Fsp-Cre; Smo^{fl}*, and Mist1- *Mist1-Kras^{G12D}; Smo^{fl}* mice (Figure 2D). Collectively, this data shows that we can generate pure populations of murine pancreatic CAFs and that *Smo^{fl}* alleles are excised in fibroblasts in *Mist1-Kras^{G12D}; Fsp-Cre; Smo^{fl}* mice, leading to decreased SMO protein.

Loss of Smo in Pancreatic Cancer Associated Fibroblasts Increases Premalignant Lesions in the Mist1-Kras^{G12D} Murine Pancreatic Tumor Initiation Model.

In order to show the effect of *Smo* loss in pancreatic fibroblasts on tumorigenesis events, we monitored pancreatic tumor development by H&E staining from *Mist1-Kras^{G12D}; Fsp-Cre; Smo^{fl}*, and *Mist1-Kras^{G12D};Smo^{fl}* mice at 23 weeks of age. ADM staining showed that *Mist1-Kras^{G12D}; Fsp-Cre; Smo^{fl}* mice have significantly increased acinar-to-ductal metaplastic (ADM) lesions as measured by ducts that are double positive for epithelial cell marker cytokeratin 19 (CK19) as well as acinar cell marker amylase (Figure 3A). Additional histological analysis showed that mice lacking *Smo* in pancreatic CAFs have enhanced onset of Pancreatic Intraepithelial Neoplastic (PanIN) lesions relative to *Smo*-intact age-matched controls (Figure 3B). In order to explain the increase in premalignant lesions, we stained pancreatic sections for the fibroblast marker smooth muscle actin (SMA) and the cellular proliferation marker Ki67 and showed that *Smo* fibroblast deleted mice had increased percentage of proliferating ductal cells (Figure 3C). Thus, these results indicate that fibroblast specific deletion of *Smo* in the pancreas leads to a significant acceleration in premalignant PanIN and ADM lesion formation due to an increase in the proliferation potential of the pancreatic epithelium.

Smo Deletion Leads to Loss of PTEN in Pancreatic Cancer Associated Fibroblasts

To understand how deleting *Smo* may change the proliferation potential of the adjacent tumor cell, we isolated CAFs from *Mist1-Kras^{G12D}; Fsp-Cre; Smo^{fl}*, and *Mist1-Kras^{G12D};Smo^{fl}* pancreata and performed microarray (Figure 4A). Gene expression analysis followed by Gene Set Enrichment Analysis (GSEA) determined that *Smo* deletion significantly altered the PI3K pathway within fibroblasts (Figure 4B). Heatmaps depicting to top up- and down-regualted genes in each genotype are shown (Figure 4C). Altered PI3K signaling in KCS fibroblasts was validated by Western blot analysis showing increased phosphorylation of AKT at Serine-473 (Figure 4D). Because AKT signaling was activated, we also analyzed the expression of the PTEN tumor suppressor, a key negative regulator of this pathway. PTEN protein levels were significantly decreased in *Smo*-deleted fibroblasts (Figure 4D).

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interest.

Acknowledgments

Figure 4A image of GeneChip® Scanner 3000 7G adapted from Affymetrix support website. We graciously thank the Solid Tumor Pathology Core at OSU for their technical assistance in processing tumor samples.

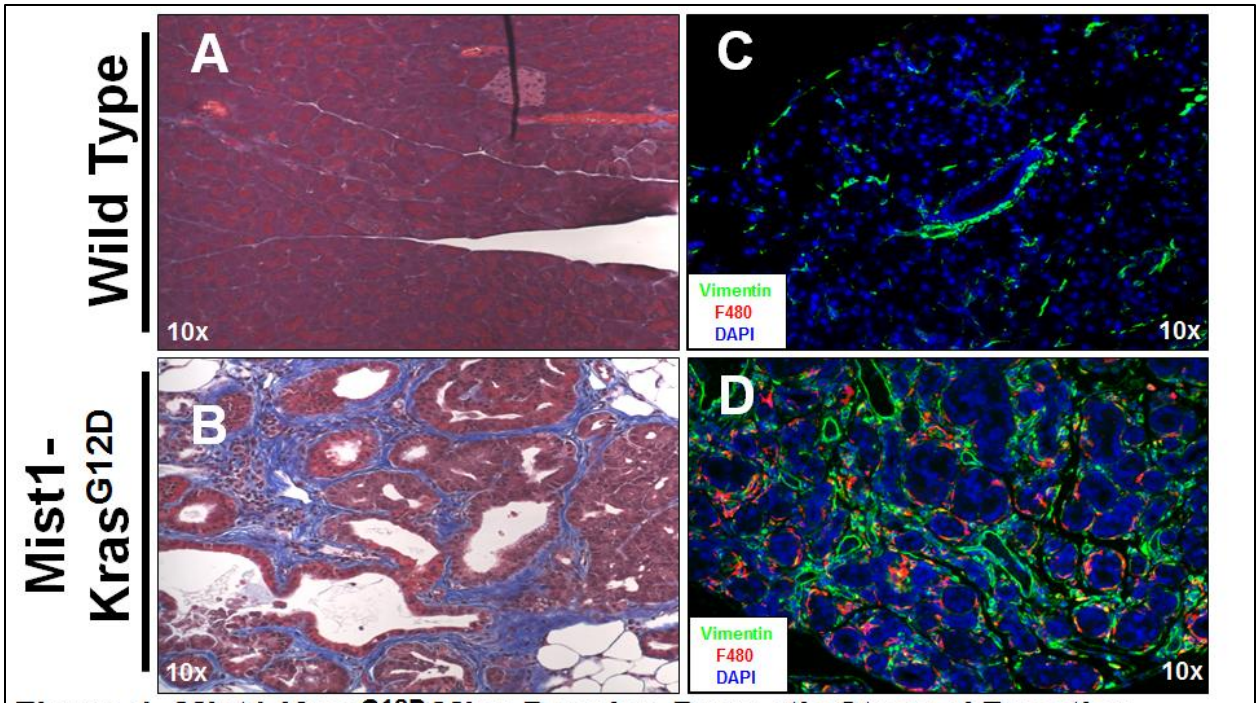

Figure 1. Mist1-Kras^{G12D} Mice Develop Dramatic Stromal Reaction.

(A,B) Trichrome staining showing Mist1-Kras^{G12D} mice develop PanIN and ADM lesions with significant stromal invasion at 23 weeks; histology resembles human condition.

(C,D) Mist1-Kras^{G12D} mice pancreata show increased vimentin+ fibroblast and F4/80+ macrophage populations.

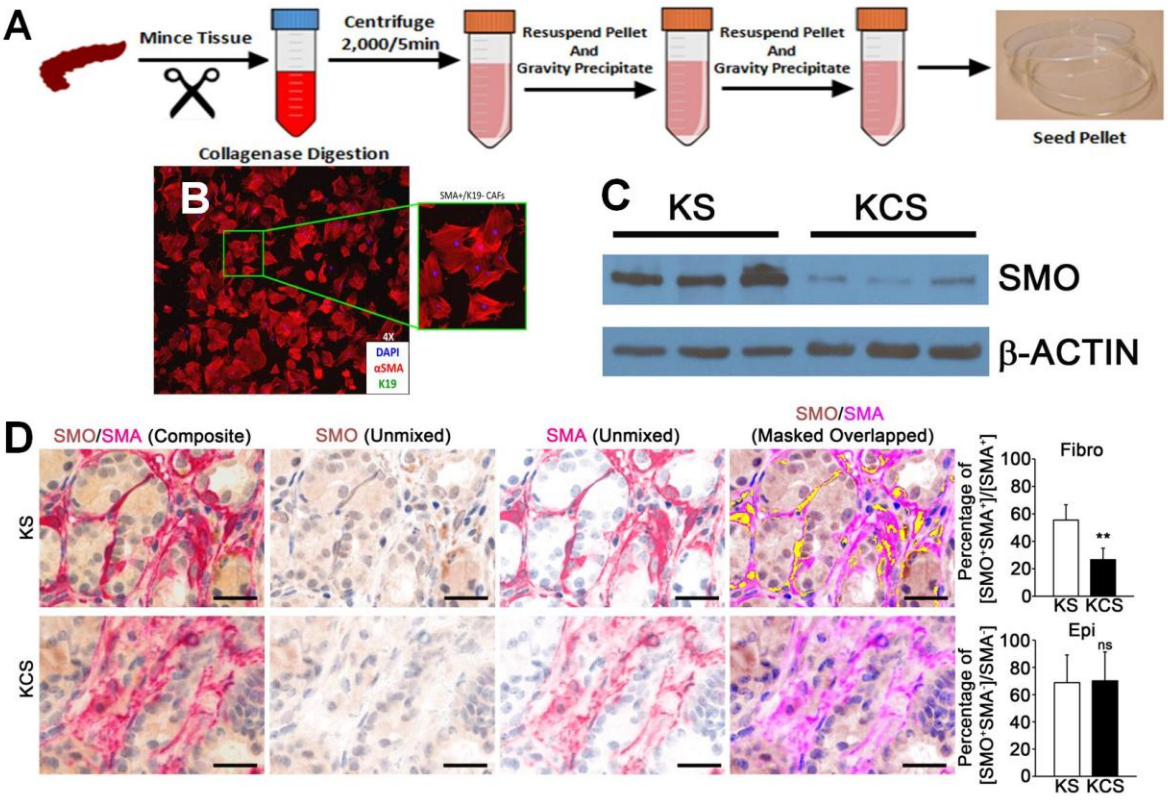

Figure 2. *Smo*^{loxP} Allele is Efficiently Deleted in pancreatic CAFs.
(A) Procedure for isolating pancreatic CAFs. (B) Primary cultures stain positive for fibroblast marker SMA (red) and lack epithelial marker K19 (green). (C) Western Blot Analysis showing decrased *SMO* protein expression in Smo conditional knockout (CKO) fibroblasts. (D) SMA/SMO staining of *Smo*-intact and *Smo*-deleted pancreata. (* $p<0.05$)

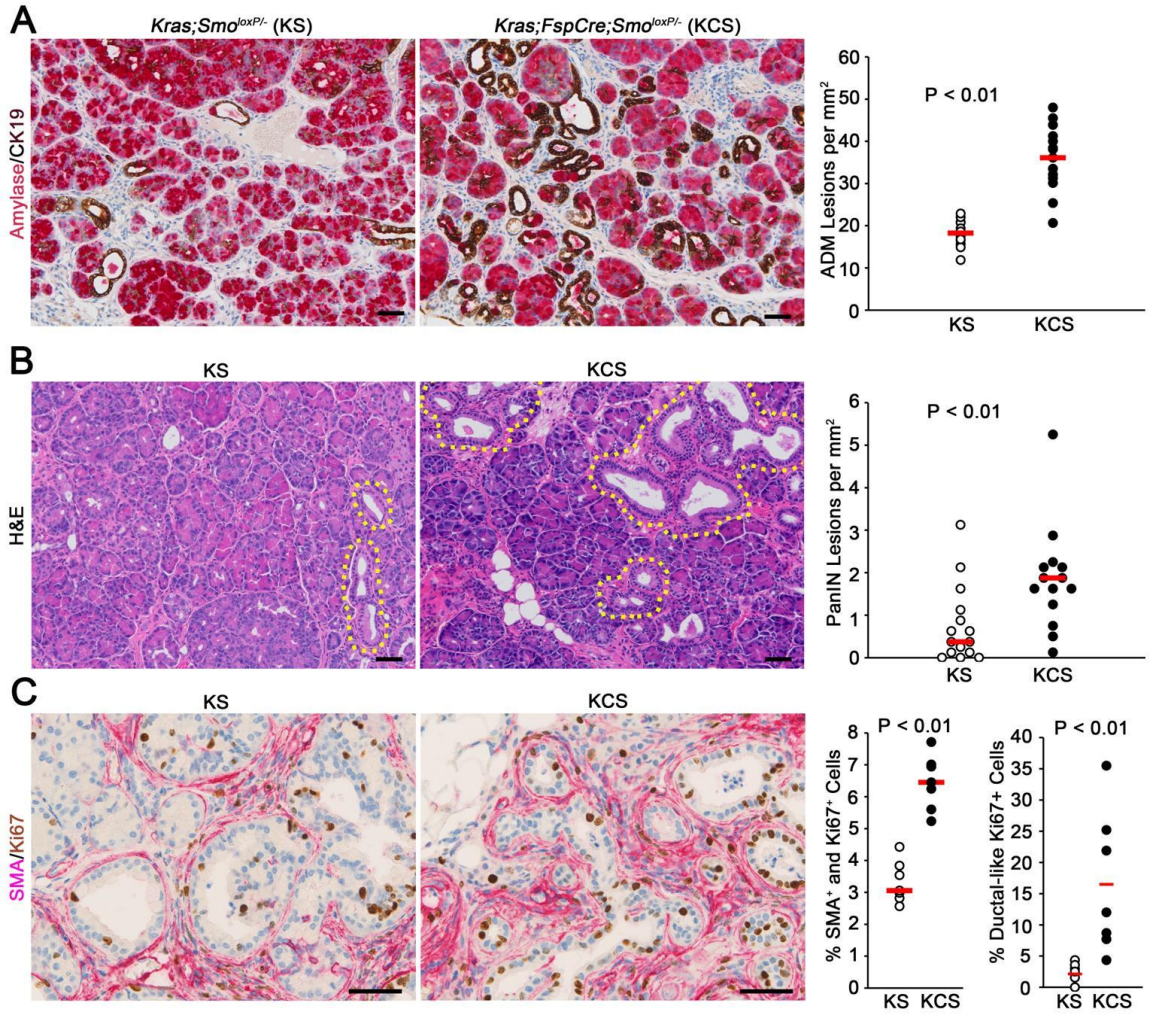

Figure 3.

(A) Dual IHC staining for acini marker amlyase (red) and ductal marker (brown) shows that Kras;FspCre;Smo mice have increased acinar-to-ductal metasplasic (ADM, amylase/K19 double positive) lesions. (B) H&E staining indicates that Kras;FspCre;Smo mice have increased PanIN lesions. (C) Dual IHC staining for SMA (red) and proliferation marker Ki67 (green) showing that Kras;FspCre;Smo mice have increased percentage of proliferating fibroblast and ductal cells. Red bars depict averages; *p<0.05

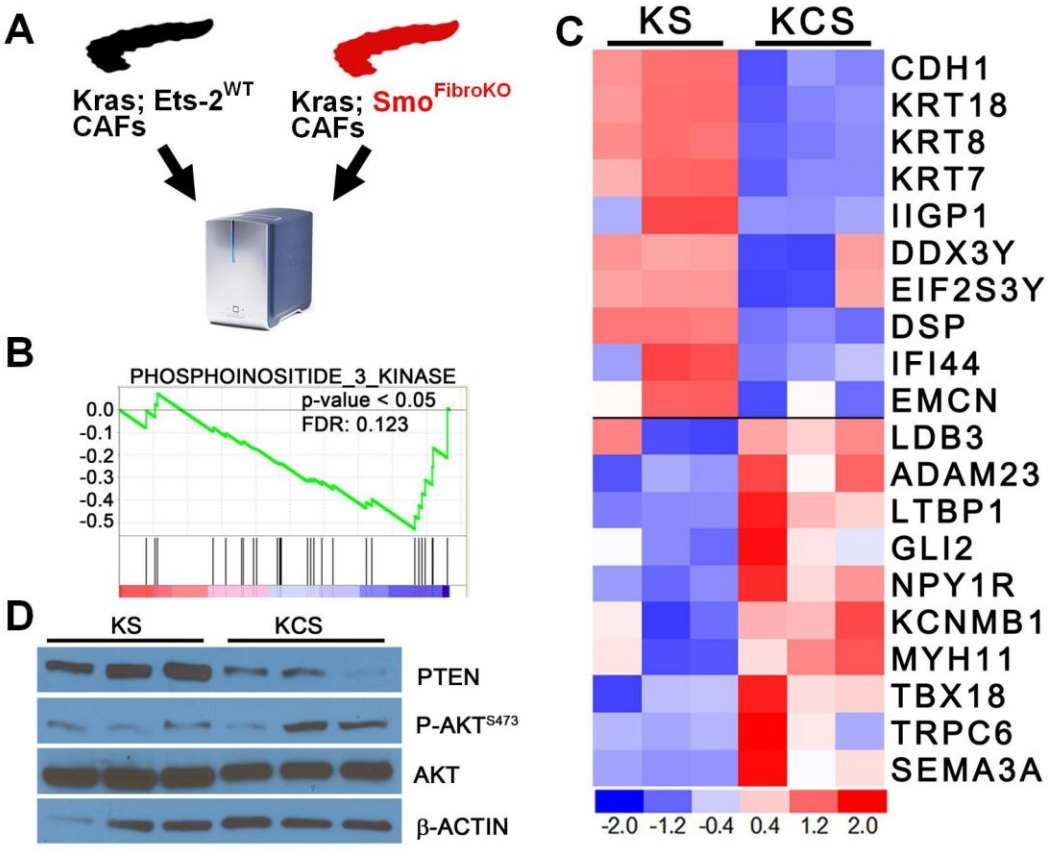

Figure 4.

(A) Cartoon depiction of microarray experiment between *Smo*^{WT} and *Smo*^{FibroKO} CAFs[4]. (B) Gene Set Enrichment Analysis (GSEA) plot showing mis-regulation of PI3K pathway in Smo^{FibroKO} pancreatic CAFs. (C) Heatmap depicting top up- and down-regulated genes. (D) Western blot showing loss of PTEN and activation of AKT in Smo-deleted fibroblasts.

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